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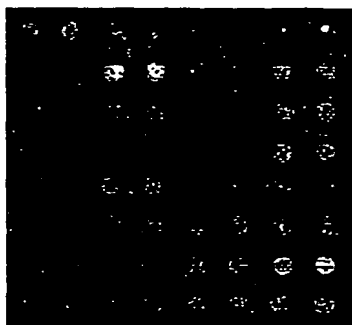
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(57) Abstract: The present invention relates to diagnosis kit for Mycobacterium species identification and drug-resistance detection and manufacturing method thereof, which can discriminate a Mycobacterium Tuberculosis rpoB gene point mutation relating to the Mycobacterium species identification and drug-resistance swiftly, exactly and in large quantities using an oligonucleotide chip. The diagnosis kit for Mycobacterium species identification and drug-resistance detection in accordance with the present invention consists of an oligonucleotide chip including a Mycobacterium tuberculosis Mycobacterium species identification probe and a drug-resistance detection probe of a complex probe, a Mycobacterium tuberculosis rpoB gene, and a fluorescent material containing a biotin-binding protein so as to detect hybridization of amplified products of a specimen marked as biotine and the corresponding probe.

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**DIAGNOSIS KIT FOR MYCOBACTERIUM SPECIES  
IDENTIFICATION AND DRUG-RESISTANCE DETECTION AND  
MANUFACTURING METHOD THEREOF**

5

**DESCRIPTION**

**Technical Field**

The present invention relates to a diagnosis kit for Mycobacterium species identification and drug-resistance detection and a manufacturing method thereof, and more specifically, to a diagnosis kit for Mycobacterium species identification and drug-resistance detection in which point mutations of Mycobacterial rpoB gene related to the drug-resistance can be discriminated speedily and accurately in large quantity by using an oligonucleotide chip, and a manufacturing method thereof.

15

**Background Art**

About two million people die from tuberculosis worldwide each year. The increase in immigration, the spread of HIV/AIDS, and the emergence of drug-resistance strains are enhancing the mortality of tuberculosis. AIDS patients and newborns with weak immune system can develop tuberculosis from not only Mycobacterium tuberculosis infection but also MOTT (Mycobacterium other than tuberculosis) infection, particularly, Mycobacterium avium-intracellulare (MAI), Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium kansasii, Mycobacterium xenopi, Mycobacterium marinum, Mycobacterium scrofulaceum, and Mycobacterium szulgai.

Tuberculosis is normally treated by chemotherapy with various anti-tuberculosis drugs. Since there are numerous different strains of Mycobacteria with diverse drug-susceptibility, detection and identification of the causative bacterium is important for the effective treatment.

For the diagnosis of tuberculosis, chest X-ray examination and sputum

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test are commonly used. However, chest X-ray can often misdiagnose an already-cured inactive tuberculosis or other types of chest diseases as tuberculosis. Sputum test can be typically performed by the following two methods: sputum smear microscopy in which sputum of a patient is thinly spread  
5 on a slide, and then the bacteria are selectively stained for microscopic observation, and culture method in which the bacteria are cultured under physiological conditions for visual observation. Sputum smear microscopy is relatively simple and rapid, but cannot easily discriminate *Mycobacterium tuberculosis* from MOTT. Detection and identification of the mycobacterium strains have been done by the culture method, but it takes at least 6-8 weeks due  
10 to the slow growth rate of the bacteria. During this period, a combination of primary anti-tuberculosis drugs is used, which may result in therapeutic failure and drug-resistance. In fact, increasing numbers of strains have been determined to be resistant to primary anti-tuberculosis drugs such as isoniazid  
15 (INH), rifampin (RIF), streptomycin (STR), ethambutol (EMB), pyrazinamide (PZA).

Therefore, in order to achieve effective control and treatment of tuberculosis, a necessity of rapidly identifying the causative bacterium at the initial stage of infection has been increasingly recognized. Recently, molecular  
20 biological techniques by which mycobacterial species identification can be made within a relatively short time have been devised. These methods use species-specific sequences of the mycobacterial genes such as IS6110 as the target DNA. However, the IS6110 gene has different copy numbers in various mycobacterial species and does not present in some species. Several reports indicate that the  
25 IS6110 gene is not mycobacterium-specific. Therefore, the use of IS6110 as target DNA may cause false-positive or false-negative results. The highly polymorphic region of 16S rRNA DNA has also been used for species identification since it shows species-specific polymorphism. Several commercial products have been developed based on this method: Accuprobe  
30 (Gen-probe, San Diego, CA, U.S.A.) which can be applied directly to a specimen,

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and AMTD (Gen-probe, San Diego, CA, U.S.A.) and Amplicor MTB (Roche Diagnostics Systems, Somerville, NJ, U.S.A.) in which target DNA are PCR (polymerase chain reaction) amplified prior to analysis to enhance sensitivity. However, the 16S rRNA gene occasionally has different copies in a single strain, and this could complicate accurate species determination. Moreover, in order to assess the drug-resistance, an additional molecular biological analysis on the rpoB gene has been necessary.

Hereinafter, point mutations of *Mycobacterium tuberculosis* related to drug-resistance are described in detail. Resistance against RIF, a primary anti-tuberculosis drug, is known to be related to the mutations of rpoB gene encoding RNA polymerase  $\beta$ -subunit. More than 30 different mutations identified so far have shown to be concentrated in the 81bp core region of the 3,534bp rpoB gene (Amalio Telenti, Paul Imboden, Francine Marchesi, Douglas Lowrie, Sterwart Cole, M. Joseph Colston, Lukas Matter, Kurt Schopfer, and Thomas Bodmer, "Detection of rifampin-resistance mutation in *Mycobacterium tuberculosis*" THE LACET, Vol.341, pp. 647-650, 1993).

Table 1 illustrates point mutations occurring in codons 507 through 533 of the *M. tuberculosis* rpoB gene. As illustrated in Table 1, point mutations occur more frequently in codons 513, 516, 526, and 531, which are also observed more prevalently than the others.

[Table 1]

CODON	VARIANT NUCLEOTIDE (AMINO ACID CHANGE)
510	CAG(Gln) > CAT(His)
511	CTG(Leu) > CCG(Pro), CGG(Arg)
512	AGC(Ser) > ACC(Thr), CGC(Arg)
513	CAA(Gln) > CTA(Leu), AAA(Lys), CCA(Pro)
515	ATG(Met) > ATA(Ile)
516	GAC(Asp) > GTC(Val), TAC(Tyr), GAG(Glu), GGC(Gly)
521	CTG(Leu) > ATG(Met)
522	TCG(Ser) > TTG(Leu)
526	CAC(His) > TAC(Tyr), GAC(Asp), CGC(Arg), CTC(Leu), CCC(Pro), CAA(Gln), AAC(Asn), CAG(Gln)
531	TCG(Ser) > TTG(Leu), TGG(Trp), TGT(Cys), CAG(Gln)

Rifabutin (abbreviated as RIB), a spiro-piperidyl derivative of rifamycin S, is used to prevent MAC infection in AIDS patients (James M. Musser, "Antimicrobial Agent Resistance in Mycobacteria: Molecular Genetic Insights", Clinical Microbiology Review, Vol. 8, No. 4, pp. 496-514, 1995). Resistance

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against RIB is also related to the point mutations of the *rpoB* gene. However, the drug-susceptibility of a mutation displays difference between RIF and RIB: Several mutations showing resistance to RIF have determined to be susceptible to RIB. Mutation showing resistance to both RIF and RIB were also reported  
5 (Della Bruna C., G. Schiopacassi, D. Ungheri, D. Jabes, E. Morvillo, and A. Sanfilippo, "A new spiro-piperidyl rifamycin: in vitro and in vivo studies", J. Antibiot. Vol. 36, pp. 1502-1506, 1983, Dessen A., A. Quenmard, J. S. Blanchard, w. R. Jacobs, Jr., and J. C. Sacchettini, "Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*" Science, Vol. 267, pp.  
10 1638-1641, 1995). RIB has often been used to treat RIF-resistant infection, which were effective in many cases (Gillespie S. H., A. J. Baskerville, R. N. Davidson, D. Felmingham, and A. D. M. Bryceson, "The serum rifabutin concentrations in patient successfully treated for multi-resistant *Mycobacterium tuberculosis* infection", J. Antimicrobi. Chemother. Vol. 33, pp. 661-674, 1990).

15 In addition, the species-specific characteristics of *rpoB* gene have been used for mycobacterial species identification. Korean Patent Registration No. 234975 discloses a method for the detection and identification of mycobacterial strains by PCR-RFLP (restriction fragment length polymorphism after polymerase chain reaction) assay using a set of novel PCR primers that  
20 specifically amplifies the *rpoB* gene of mycobacterial species. Also, Korean Patent Registration No. 285254 discloses a method of detecting and distinguishing *Mycobacterium tuberculosis* complex and MOTT by using primers that can amplify *rpoB* gene fragments of *Mycobacterium tuberculosis* complex and MOTT in a respectively different size.

25 However, the above-described methods require bacterial culture step prior to PCR amplification, limit the kind of species or the number of samples that can be analyzed, and are laborious having a PCR step followed by a restriction enzyme treatment step and/or an electrophoresis step.

In the Korean Patent Registration No. 285253, a method of detecting  
30 rifampin-resistance in *Mycobacterium tuberculosis* with a nested PCR-SSCP

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(polymerase chain reaction-single strand conformational polymorphism) and a single-step nested PCR-SSCP. This method include two PCR and polymorphism assay steps and therefore it takes about four days to determine rifampin-resistance.

5 PCT International Publication No. WO97/29212 by Affymetrix discloses a technology of a sequencing DNA chip of rpoB gene. With this method, the entire base sequences of 700bp in the rpoB gene should be analyzes to obtain clinically important information such as species identity and drug-resistance.

10 **Disclosure of the Invention**

To solve the above problems, it is an object of the present invention to provide a diagnosis kit for Mycobacterium species identification and drug-resistance detection in which features relating to both the Mycobacterium species identification and the drug-resistance detection can be discriminated speedily and  
15 accurately in large quantity.

It is another object of the present invention to provide a method of manufacturing a diagnosis kit for Mycobacterium species identification and drug-resistance detection in which features relating to both the Mycobacterium species identification and the drug-resistance detection can be discriminated speedily and  
20 accurately in large quantity.

It is still another object of the present invention to provide a set of primers of a polymerase chain reaction (PCR) for faster Mycobacterium species identification by enabling efficient PCR amplification of rpoB gene fragment (157bp) of numerous species directly from uncultured specimens including  
25 sputum samples.

It is yet another object of the present invention to provide experimental conditions for polymerase chain reaction (PCR) for efficient amplification of the 157bp fragment of rpoB gene from various Mycobacteria.

To accomplish the above objects of the present invention, there is  
30 provided a diagnosis kit for Mycobacterium species identification and drug-

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resistance detection comprising: an oligonucleotide chip including species identification probes comprised of species-specific DNA sequences of Mycobacterial rpoB gene, Mycobacterial drug-resistance detection probes comprised of one or more modified codons of Mycobacterial rpoB gene, and  
5 contrast group probes comprised of wild-type sequences corresponding to each drug-resistance detection probe; and a method for detecting the hybridization of the oligonucleotide chip and the specimen DNA.

By the above construction, drug-resistance detection as well as Mycobacterium species identification can be simultaneously performed. In  
10 particular, since Mycobacterial rpoB gene point mutations related to drug-resistance can be discriminated by comparing the intensity of hybridization signal of the drug-resistance detection probe with that of the contrast group probe, and species identification can be made by comparing the hybridization intensities of the species-specific probes, the Mycobacterial species identification and the  
15 drug-resistance detection can be performed speedily and accurately in large quantity.

According to a specific embodiment of the present invention, in said diagnosis kit for Mycobacterial species identification and drug-resistance detection, the drug-resistance detection probe comprises one or more modified  
20 codons discovered in 507-533 codons of rpoB gene.

By the above construction, since one or more modified codons discovered in 507-533 codons in which point mutations frequently occur are used as a probe, drug-resistance can be discriminated speedily and accurately with only part of the Mycobacterial rpoB gene.

25 According to a more specific embodiment of the present invention, in said diagnosis kit for Mycobacterial species identification and drug-resistance detection, the drug-resistance detection probes are rifampin-resistance probes and/or rifabutin-sensitive probes.

By the above construction, it can be discriminated speedily and  
30 accurately whether a specimen has rifampin-resistance and/or rifabutin

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susceptibility.

According to another embodiment of the present invention, there is provided a method of manufacturing a diagnosis kit for *Mycobacterium* species identification and drug-resistance detection comprising the steps of: (a) 5 modifying species identification probes comprised of species-specific DNA sequences of *Mycobacterial* *rpoB* gene, *Mycobacterial* drug-resistance detection probes comprised of one or more modified codons of *Mycobacterial* *rpoB* gene, and contrast group probes comprised of wild-type sequences corresponding to each drug-resistance detection probe to contain an amine group on the 5' 10 terminal; (b) inducing an aldehyde group on glass; and (c) fabricating an oligonucleotide chip by immobilizing the modified probes on the glass by Schiff base reaction.

By the above construction, the diagnosis kit having the advantage of simultaneously discriminating *Mycobacterium* species identity and drug- 15 resistance speedily and accurately in large quantity, can be easily fabricated.

According to another aspect of the present invention, a set of PCR primers for *Mycobacterium* species identification comprises DGR8 and DGR9 having base sequences by which *Mycobacterial* *rpoB* gene fragments (157bp) are specifically amplified.

20 In the case that the primer set is used, DNA isolated from uncultured clinical specimens can be used as a template of a polymerase chain reaction, and the *rpoB* gene fragment (157bp) of 44 species belonging to *Mycobacterium* genus can be efficiently amplified. Accordingly, the *Mycobacterium* species identification and the drug-resistance detection can be performed within a short 25 time, for example, approximately in six hours. The PCR products obtained by using the primer set are DNA of 157bp size that can be used as a target DNA of oligonucleotide chip analysis.

In a method for *Mycobacterium* species identification and drug-resistance detection according to still another aspect of the present invention, a fragment of 30 *rpoB* gene in a specimen is PCR amplified under the reaction conditions such as



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an annealing temperature of 64°C, a reaction cycle of 40, and a primer concentration of 100pmol with primers DGR8 and DGR9, and then the amplified product is used for Mycobacterial species identification and drug-resistance detection by using said diagnosis kit according to the present invention.

5 DNA chip technology developed in early 1980s presents a method of providing a large amount of genetic information at a time. This method has been used to investigate gene expression, mutation and polymorphism of human or bacterium genome.

The present invention utilizes the DNA chip technology that enables  
10 rapid genetic analysis, and provides a diagnosis kit for Mycobacterium species identification and drug-resistance detection and a manufacturing method thereof, in which the Mycobacterium species identification and the point mutations of Mycobacterium tuberculosis related to drug-resistance can be discriminated.

In the present invention, mutations occurring in the rpoB gene of  
15 Mycobacterium tuberculosis have been investigated to identify drug-resistant strains, based on the relationship between drug-susceptibility and rpoB gene mutation described in the background art. More specifically, the rpoB gene fragment (157bp) including the 81bp core region where Mycobacterial point mutations occur most frequently is PCR amplified using a particular primer set,  
20 and then the amplified DNA is hybridized with an oligonucleotide chip, to thereby detect point mutations for drug-resistance determination.

The method using said oligonucleotide chip includes a step of amplifying target DNA from clinical specimens by PCR. In order to successfully apply the method to clinical samples, PCR should be performed with highly efficient  
25 primers.

In Korean Patent Application No. 2000-0029369, biotin-TR8 and TR9 that have been devised by using only the rpoB gene base sequence of Mycobacterium tuberculosis are used as primers. The DNA amplified by using the primers is 157bp fragment containing the 81bp core region where point  
30 mutations related to rifampin-resistance occur most frequently. However, the

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amplification efficiency of biotin-TR8/ TR9 primers is not sufficient for uncultured specimens such as sputum and therefore the analysis time with these primers exceeds 6-8 weeks due to bacterial culture step. MOTT may not be amplified even from cultured samples due to the low efficiency of these primers.

5 In other words, a rapid analysis on the species identity and drug-resistance cannot be accomplished without DNA amplification directly from sputum samples.

Thus, the inventors combine base sequences of the rpoB gene of forty-three different species of Mycobacteria as well as Mycobacterium tuberculosis, to thereby devise a new primer set. When this primer set is used, isolated DNA  
10 from uncultured specimens can be used as a template for PCR, and a specific rpoB gene sequence of forty-three bacterial species as well as Mycobacterium tuberculosis can be amplified by a single PCR step. When the amplified product is applied to a DNA chip, the Mycobacterium species identification and rpoB mutation detection can be simultaneously performed within a short period  
15 of time.

#### **Brief Description of the Drawings**

FIG. 1 is a flowchart view illustrating a process of manufacturing an oligonucleotide chip for Mycobacterium species identification and drug-  
20 resistance detection according to the present invention;

FIG. 2 is a pictorial view showing a result in which rpoB gene is amplified using biotin-TR8 and TR9 primers and then undergone an electrophoresis in 2% agarose gel, according to the present invention;

FIGs. 3A through 3J are pictorial views showing fluorescent images  
25 obtained by hybridization of PCR amplified clinical samples using biotin-dUTP with the oligonucleotide chip, according to the present invention;

FIG. 4 is a view of an array format indicating the probe positions of the oligonucleotide chip used in the FIGs. 3A through 3J views;

FIG. 5 is a diagram showing an array format of an oligonucleotide chip  
30 using probes of SEQ ID NO 5 through 12 according to the present invention;

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FIGs. 6A through 6C are pictorial views showing the experimental results of Mycobacterial species identification using the oligonucleotide chip of FIG. 5;

FIG. 7 is a diagram showing an array format of an oligonucleotide chip in which probes of base sequences of SEQ ID NO 41 through 46 detecting additional rpoB gene mutations related to rifampin-resistance in Mycobacterium tuberculosis are included;

FIGs. 8A through 8D are pictorial views showing the diagnostic results of clinical isolates regarding rifampin-resistance and rifabutin-susceptibility using the oligonucleotide chip of FIG. 7;

FIG. 9A is a pictorial view showing a 2% agarose gel DNA band image of PCR amplified products using PCR conditions disclosed in the prior application of the inventors, i.e. an annealing temperature of 63 °C and 35 cycles with biotin-TR8 and TR9 primers of 50pmol;

FIG. 9B is a pictorial view showing a 2% agarose gel image of PCR amplified products using the PCR conditions according to the present invention, i.e. an annealing temperature of 64 °C and 40 cycles with biotin-DGR8 and DGR9 primers of 100pmol;

FIG. 10 is a pictorial view showing a 2% agarose gel image of eleven sputum specimens PCR amplified by using biotin-DGR8 and DGR9 primers under the optimized condition according to the present invention; and

FIGs. 11A through 11B are pictorial views showing the diagnostic results of sputum specimens regarding rifampin-resistance using the oligonucleotide chip of FIG. 7.

#### **Best Mode for Carrying Out the Invention**

Preferred embodiments of the present invention will be described below in detail with reference to the accompanying drawings. However, the following embodiments are nothing but embodiments for demonstrating structures and effects of the present invention. Thus, the present invention is not limited to the following embodiments.

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Embodiment 1-1: Mycobacterial genomic DNA

Mycobacterial genomic DNA extracted from ten clinical isolate cultures were provided after sequencing from the Department of Respiratory Diseases, Asan Medical Center (Seoul, Korea), and additional Mycobacterial genomic  
5 DNA were obtained from ATCC (Manassas, VA USA).

Embodiment 1-2: Fabrication of oligonucleotide probes and primers

Table 2 illustrates sequences of oligonucleotide probes and primers. Each of biotin-TR8 and TR9 and probes used were custom-synthesized from Bionics (Seoul, Korea). All other reagents were first-grade.

10 [Table 2]

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Probes or Primers	Sequences
<u>Tuberculosis bacteria group probes</u>	
Mc1	5'Amine-T10-tcttcggcaccagccag 3'
Mc2	5'Amine-T10-tcttcggaaccagccag 3'
Mc5	5'Amine-T10-tcttcggaacgtcgag 3'
Mex	5'Amine-T10-tcttcggaacctcgag 3'
<u>Mycobacterium species identification probes</u>	
Ms1	5'Amine-T10-ggtctgtcacgtgagcgtg 3'
Ms2	5'Amine-T10-ggtctgtcccgtgagcgtg 3'
Ms3	5'Amine-T10-ggtctgtcgcgtgagcgtg 3'
Ms4	5'Amine-T10-ggtctgtcccgggagcgtg 3'
Ms5	5'Amine-T10-ggtctgtcccgcgagcgtg 3'
Ms6	5'Amine-T10-ggtctgtcgcgcgagcgtg 3'
Ms7	5'Amine-T10-ggtctgacctgagcgtg 3'
Ms8	5'Amine-T10-ggtctgagccgggagcgtg 3'
<u>Wild-type probes</u>	
F2	5'Amine-T10-cagccagctgagccaat 3'
F3	5'Amine-T10-gctgagccaattcatgg 3'
F4	5'Amine-T10-attcatggaccagaaca 3'
F5	5'Amine-T10-tggaccagaacaaccg 3'
F6	5'Amine-T10-caacccgctgtcgggt 3'
F7	5'Amine-T10-ggttgaccacaagcgc 3'
<u>Rifampin-resistance probes</u>	
mt511	
mt513	5'Amine-T10-cagccagctgagccaat 3'
mt516a	5'Amine-T10-gctgagccaattcatgg 3'
mt516b	5'Amine-T10-attcatggaccagaaca 3'
mt518	5'Amine-T10-attcatgtaccagaaca 3'
mt521	5'Amine-T10-tggaccagcacaaccg 3'
mt522	5'Amine-T10-caacccgatgtcgggt 3'
mt526a	5'Amine-T10-caacccgctgtgggt 3'
mt526b	5'Amine-T10-ggttgacctacaagcgc 3'
mt526c	5'Amine-T10-ggttgaccgacaagcgc 3'
mt531	5'Amine-T10-ggttgaccgcaagcgc 3'
mt516c	5'Amine-T10-ccgactgtggcgctgg 3'
mt526d	5'Amine-T10-attcatggaccagaaca 3'
mt526e	5'Amine-T10-ggttgaccacaagcgc 3'
mt526f	5'Amine-T10-ggttgaccgccaagcgc 3'
mt526g	5'Amine-T10-ggttgacctgcaagcgc 3'
mt526h	5'Amine-T10-ggttgaccagaagcgc 3'
cf) Rifabutin-sensitive probes :	5'Amine-T10-ggttgaccggcaagcgc 3'
<u>Primers</u>	
Bio-TR 8	5'biotin-tgcacgtcgcggacctcc 3'
TR 9	5'tcgcgcgatcaaggagt 3'
<u>Mycobacterium species identification additional probes</u>	
MTAB	5'Amine-T10-gcagctgagccaattcat 3'
MF	5'Amine-a10-cgacgtcgcagctgtcg 3'

According to the above order illustrated in Table 2, SEQ ID NOs 1-40 are assigned.

As illustrated in Table 2, an oligonucleotide chip for investigating point mutation of *rpoB* gene is devised including seven wild-type probes, seventeen RIF-resistance probes, and eight RIB-sensitive probes:

As a result, seventeen RIF-resistance probes of SEQ ID NOs 20-36 can  
 5 discriminate the mutations of codon 511 (Leu >Pro), codon 513 (Gln >Pro),  
 codon 516 (a: Asp >Val, b: Asp >Tyr, c: Asp >Glu), codon 518 (Asn >His),  
 codon 521 (Leu >Met), codon 522 (Ser >Leu), codon 526 (a: His >Tyr, b:  
 His >Asp, c: His >Arg, d: His >Asn, e: His >Ala, f: His >Cys, g: His >Gln, h: His  
 >Gly), and codon 531 (Ser >Leu).

10 Eight RIB-sensitive probes of SEQ ID NOs 22, 23 and 31-36 among  
 seventeen RIF-resistance probes can discriminate the mutations of codon 516 (a:  
 Asp >Val, b: Asp >Tyr, c: Asp >Glu), and codon 526 (d: His >Asn, e: His >Ala,  
 f: His >Cys, g: His >Gln, h: His >Gly).

Strains having mutation type 516a and 516b have shown conflict with  
 15 respect to RIB-susceptibility as indicated in many reports (James M. Musser,  
 "Antimicrobial Agent Resistance in Mycobacteria: Molecular Genetic Insights",  
 Clinical Microbiology Review, Vol.8, No. 4, pp. 496-514, 1995). The current  
 format of the oligonucleotide chip includes 516a and 516b probes in RIB-  
 sensitive probes.

20 Probes Mc1, Mc2, and Mc5 of SEQ ID NOs 1-3 devised in order to  
 discriminate Mycobacterium species from other bacteria are mixed and affixed as  
 a single spot on an oligonucleotide chip. From the positive reaction on the  
 probes, Mc1 can detect *M. tuberculosis*, *M. africanum*, *M. asiaticum*, *M. bovis*  
 (2), *M. gastri*, *M. avium* (2), *M. celatium* (2), *M. genavense*, *M. gordonae*, *M.*  
 25 *haemophilum*, *M. interjectum*, *M. intermedium*, *M. intracellulare*, *M. kansasii*, *M.*  
*leprae*, *M. malmoense*, *M. scrofulaceum*, *M. szulgai*, *M. xenopi*, *M. terrae*, *M.*  
*triviale*, *M. nonchromogenicum*, *M. aurum*, *M. chitae*, *M. peregrinum*, *M. phlei*,  
*M. smegmatis*, *M. thermoresistibile*, and *M. vaccae*, Mc2 can detect pathogenic  
 or non-pathogenic but clinically important *M. marium*, *M. shimoidei*, *M.*  
 30 *ulcerans*, *M. abscessus*, and *M. chelonae*, and Mc5 can detect *M. fortuitum*.

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Also, probe Mex of SEQ ID NO 4 detecting *Corynebacterium diphtheriae* which is not mycobacteria but belongs to similar but different genus as a contrast group, is added in order to compare hybridization signals with each other, to thereby enable a more accurate determination on whether or not the strain under examination belongs to Mycobacteria.

Eight Mycobacterial species identification probes of SEQ ID NO 5 through 12 including partial sequences of Mycobacterial species-specific *rpoB* gene have been added in order to perform Mycobacterial species identification in more detail, in addition to probes which can discriminate Mycobacteria from other similar bacteria. In the case of the Mycobacterium species identification probes, the Ms1 probe can detect *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. gastri*. The other probes have been devised to detect *M. asiaticum* (Ms2), *M. simiae* (Ms2), *M. aurum* (Ms2), *M. senegalense* (Ms2), *M. shimoidei* (Ms2), *M. neoaurum* (Ms2), *M. terrae* (Ms2), *M. gordonae* (Ms2), *M. haemophilum* (Ms2), *M. szulgai* (Ms2), *M. intracellulare* (Ms2), *M. kansasii* (Ms2), *M. scrofulaceum* (Ms2), *M. fortuitum* (Ms2), *M. fallax* (Ms2), *M. flavescens* (Ms2), *M. peregrinum* (Ms2), *M. phlei* (Ms2), *M. vaccae* (Ms2), *M. marinum* (Ms2), *M. ulcerans* (Ms2), *M. chitae* (Ms2), *M. genevense* (Ms2), *M. smegmatis* (Ms2), *M. intermedium* (Ms3), *M. malmonense* (Ms3), *M. nonchromogenicum* (Ms3), *M. leprae* (Ms3), *M. avium* (Ms4), *M. triviale* (Ms4), *M. celatium* (Ms4), *M. interjectum* (Ms5), *M. xenopi* (Ms6), *M. chelonae* (Ms7), *M. abscessus* (Ms7), and *M. thermoresistibile* (Ms8) which are pathogenic or non-pathogenic but clinically significant and may need to be identified.

Additional species identification probes, MTAB and MF, have been devised to specifically detect *M. tuberculosis* (MTAB), *M. africanum* (MTAB), *M. bovis* (MTAB), *M. bovis BCG* (MTAB), *M. intracellulare* (MTAB), and *M. kansasii* (MTAB), *M. fortuitum* (MF), and *M. flavescens* (MF).

#### Embodiment 1-3: Fabrication of oligonucleotide chip

FIG. 1 is a flowchart view illustrating a process of fabricating an oligonucleotide chip for Mycobacterium DNA identification according to the

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present invention. As shown in FIG. 1, the oligonucleotide chip fabrication process includes: the first step (10) of modifying each of the Mycobacterium complex probes, Mycobacterium species identification probes having Mycobacterial species-specific DNA sequences of Mycobacterium rpoB gene, Mycobacterium drug-resistance detection probes including one or more modified codons of the Mycobacterium rpoB gene, and contrast group probes including wild-type sequences corresponding to each drug-resistance detection probes, to contain an amine group at the 5' terminal; the second step (20) of introducing an aldehyde group on silylated slide glass; and the third step (30) of affixing the modified probe on the slide glass by Schiff base reaction, to thereby fabricate an oligonucleotide chip.

More specifically, a particular DNA probe (see Table 2) was dissolved in  $3\times\text{SSC}$  to a concentration of  $200\text{pmol}/\mu\text{l}$ . The DNA probe solution of  $0.1\text{--}0.2\mu\text{l}$  was spotted on an aldehyde-derivatized slide glass (CEL Associates, Inc., MA, U.S.A), and reacted in a humidified incubator for 4 hours at  $37^\circ\text{C}$ . Then the slide glass was treated with 0.2% sodium dodecyl sulfate (SDS) solution for one minute, distilled water for one minute, and then with  $\text{NaBH}_4$  solution ( $0.1\text{g NaBH}_4$ ,  $30\text{ml PBS}$ , and  $10\text{ml EtOH}$ ) for five minutes. Finally, the slide glass was washed for one minute in distilled water and air-dried, and kept in a dark room at room temperature until it is used.

#### Embodiment 1-4: Polymerase chain reaction (PCR)

PCR has been performed in a total volume of  $50\mu\text{l}$  with  $10\text{mM Tris-HCl}$  ( $\text{pH } 8.3$ ),  $50\text{mM KCl}$ ,  $1.5\text{mM MgCl}_2$ ,  $1\text{U Taq polymerase}$ ,  $50\text{pmol biotin-TR8}$  and  $\text{TR9 primers}$  (see Table 2). Further,  $1\text{mM dATP}$ ,  $\text{dGTP}$ , and  $\text{dCTP}$  of  $2\mu\text{l}$  each,  $\text{dTTP}$  of  $1.5\mu\text{l}$ , and  $1\text{mM biotin-dUTP}$  of  $0.5\mu\text{l}$  were added to randomly incorporate fluorescence labeling in the resulting target DNA. Table 3 illustrates the composition of PCR mixture.



[Table 3]

PCR REACTION MIXTURE COMPOSITION	AMOUNT ( $\mu$ l)
10 x PCR buffer (MgCl <sub>2</sub> -free)	5
MgCl <sub>2</sub> (25 mM)	3
dNTPs (1 mM each)	2 (dT 1.5)
Biotin-dUTP (1 mM)	0.5
Primer 1 : Bio-TR 8 (50 pmol/ $\mu$ l)	2
Primer 2 : TR 9 (50 pmol/ $\mu$ l)	2
Taq polymerase (5 unit/ $\mu$ l)	0.2
Template DNA	1

The PCR was performed for 35 cycles under the following conditions.

- 5        -predenaturation: at 94 °C, for 5 minutes
- denaturation: at 94 °C, for 30 seconds
- primer annealing: at 63 °C, for 30 seconds
- polymerization: at 72 °C, for 45 seconds
- last extension: at 72 °C, for 5 minutes

- 10        The PCR products produced through the above process are 157bp including 81bp core region, and has been identified by electrophoresis on a 2% agarose gel.

#### Embodiment 1-5: Hybridization and Scanning

- 15        A target DNA (157bp) of 20 $\mu$ l amplified by PCR was mixed with DNase buffer of 20 $\mu$ l and DNase I (0.1U/ $\mu$ l) of 1 $\mu$ l, and treated at 0 °C for 5 minutes. The resulting target DNA was thermally treated at 99 °C for 10 minutes to inactivate the DNase I. The mixture was then treated with 3N NaOH of 4 $\mu$ l at 25 °C for 5 minutes, and then treated at 0 °C for 5 minutes with Tris-HCl (pH 7.2) of 2 $\mu$ l and 3N HCl of 4 $\mu$ l. To the above mixture, 12 x
- 20        SSPE (saline-sodium phosphate-EDTA buffer) of 50 $\mu$ l and 10% SDS of 0.5 $\mu$ l were added, and then transferred on to an oligonucleotide chip for hybridization at 40 °C for 3 hours.

- After hybridization, the oligonucleotide chip was washed with 2 x SSPE and 0.03% SDS at 25 °C for 3 minutes, with 1 x SSPE at 25 °C for 5 minutes,
- 25        and then with 0.2 x SSPE at 25 °C for 5 minutes to remove unreacted DNA. The oligonucleotide chip was dried at 25 °C, and a mixture of 3 x SSPE of 49 $\mu$ l

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and streptavidin-R-phycoerythrin of  $1\mu\ell$  was added, then treated at  $25^{\circ}\text{C}$  for 10 minutes. The stained oligonucleotide chip was washed twice with  $1\times$  SSPE for 1 minute, air-dried, and scanned with a laser fluorescence scanner (GMS 418 array scanner, TaKaRa, Japan).

5        The wild-type (wt) probes consist of seven 17mer probes devised from rpoB gene sequence corresponding to codons 507-533, including the mutation site in middle (positions 8-12) and have maximum sequence overlaps between the wild-type probes. The present invention has been devised to accurately detect frequent point mutations by comparing the signal intensities of the  
10       rifampin-resistance probes with those of the corresponding wild-type probes, and simultaneously predict all point mutations found at 507-533 codons.

Each mutant probe is designed to have the most frequent mutation site located in the middle position (8-12 positions) of the probe as in the wild-type probes, to thereby enhance detection sensitivity and enable more accurate  
15       detection by comparing the signal intensities with wild-type probes. The hybridization efficiency was increased by attaching a  $T_{10}$  spacer on each probe, which is confirmed experimentally. It is considered that the  $T_{10}$  spacer reduces steric hindrance and hence increases the hybridization efficiency of the target DNA and the probes.

20       FIG. 2 is a pictorial view showing the result in which rpoB gene fragments (157bp) of ten clinical isolates are amplified using biotin-TR8 and TR9 primers and then undergone an electrophoresis in 2% agarose gel, according to the present invention. The ten amplified rpoB gene fragments (157bp) were digested with DNase I, and then hybridized with the oligonucleotide chip. After  
25       hybridization under the optimal conditions, that is, at  $40^{\circ}\text{C}$  for 3 hours, the oligonucleotide chip was stained with streptavidin-R-phycoerythrin for subsequent fluorescence scanning at 578nm. The resulting image can be analyzed for drug-resistance by comparing the fluorescent intensities of the wild-type (wt) probes with those of the mutation (mt) probes relatively. Mutant  
30       probes and wild-type probes have identical sequence except for a few base

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producing mixed results.

L. B. Heifets has reported that strains having mutations of 511 (Leu>Pro), 516 (Asp>Tyr), 516 (Asp>Val), and 522 (Ser>Leu) are classified as low-level resistance strains with rifabutin  $MIC \leq 5 \mu g/ml$  (Heifets L. B., Antituberculosis drugs: antimicrobial activity in vitro, pp. 14-57 in L. B. Heifets (ed.), Drug susceptibility in the chemotherapy of mycobacterial infections, 1<sup>st</sup> ed. CRC Press, Boca Raton, Fla, 1991). For these cases, the drug-susceptibility test is necessary in addition to the oligonucleotide chip experiment.

In the cases of codon 526 mutants, it has been clearly determined that His>Gln, Gly, Asn, Ala, or Cys, are RIB-susceptibility whereas His>Tyr, Pro, Arg, or Asp are RIB-resistance, therefore, oligonucleotide chip experiment alone can discriminate RIB-susceptibility.

FIG. 5 is a diagram showing an array format of the oligonucleotide chip using probes Ms1-Ms8 designed for species identification. FIGs. 6A through 6C are pictorial views showing the experimental results of Mycobacterium species identification using the oligonucleotide chip of FIG. 5. In the case of Mycobacterium kansasii, Mycobacterium fortuitum, and Mycobacterium scrofulaceum, the signal of MCMix probe indicating mycobacteria appears more strongly than that of the control Mex probe, and the signal of number 2 probe is stronger than those of numbers 1, and 3-8 probes, among the species identification probes. Likewise, clinically significant 33 kinds of Mycobacteria can be identified. In the case of Mycobacterium tuberculosis, the signals of the MCMix probe and number 1 probe turn on, MOTT except for Mycobacterium gastri can be confirmed by signals on the MCMix probe and the number 2 through number 8 probes.

In the following embodiment 2, the materials used were: sixteen different species of Mycobacterial genomic DNA have been provided from the Jeju University (Jeju, Korea). DNA probes and primers were custom-synthesized from Bionics (Seoul, Korea). All other reagents were first-grade.

#### Embodiment 2-1: Fabrication of oligonucleotide probe

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Additional probes detecting mutations of the *rpoB* gene of rifampin-resistance *Mycobacterium tuberculosis* can be used with above-mentioned drug-resistance probes. Base sequences of three additional probes and their corresponding wild-type probes are shown in Table 4.

[Table 4]

PROBE	BASE SEQUENCE	DRUG-SUSCEPTIBILITY	IDENTIFICATION
Mt509	5'amine-t10- ggcaccagacagctgag-3'	RFP-R, RBU-S	509 MUTANT
Mt533	5'amine-t10- tgtcggcgccggggccc-3'	RFP-R, RBU-R	533 MUTANT
Mt524	5'amine-t10- tgtcggggtaaccac-3'	RFP-R, RBU-R	524 MUTANT
Wt509	5'amine-t10- ggcaccagccagctgag-3'	RFP-S, RBU-S	509 WILD TYPE
Wt533	5'amine-t10- tgtcggcgctggggccc-3'	RFP-S, RBU-S	533 WILD TYPE
Wt524	5'amine-t10- tgtcggggttgaccac-3'	RFP-S, RBU-S	524 WILD TYPE

By comparing fluorescent intensities of probes, mutations such as codon 509(Ser)→AGA(Arg), codon 533 CTG(Leu)→CCG(Pro), codon 524 TTG(Leu)→TTA(Leu) can be detected. In Table 4, RFP represents rifampin, RBU represents rifabutin, R represents resistance, and S represents susceptibility.

FIG. 7 is a diagram showing the array format of an oligonucleotide chip containing additional RIF-resistant probes. FIGs. 8A through 8D are pictorial views showing the diagnostic result regarding rifampin-resistance and rifabutin-sensitivity of clinical isolates using the oligonucleotide chip of FIG. 7. The clinical isolates used in the experiment were supplied from the Asan Medical Center (Seoul, Korea). Mutations in the samples were identified in the same manner as that of the embodiment 1 as #1 (516a mutant), #2 (511 mutant), #3 (516b mutant), and #4 (531 mutant), as shown in FIGs. 8A-8D. All the samples were *Mycobacteria* strains as judged by comparing Mmix probe with Mex probe. The result of DNA sequencing agreed with the oligonucleotide chip results in all cases.

#### Embodiment 2-2: Fabrication of primer

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The inventors used biotin-TR8 and TR9 primer set in the prior application, but has devised a new primer set, biotin-DGR8, DGR9, that can amplify *Mycobacterium tuberculosis* and MOTT from uncultured specimens such as sputum, with an increased efficiency compared to biotin-TR8/TR9.

5 The new primer set, biotin-DGR8, DGR9, has been devised by mixing partial base sequences of *rpoB* genes of forty-three kinds, that is, *M. africanum*, *M. asiaticum*, *M. avium*, *M. bovis*, *M. bovis* BCG strain French 1173P2, *M. celatum* strain ATCC51131, *M. celatum* strain ATCC51130, *M. gastri*, *M. genavense*, *M. gordonae*, *M. haemophilum*, *M. interjectum*, *M. intermedium*, *M.*  
 10 *intracellulare*, *M. kansasii*, *M. leprae*, *M. malmoense*, *M. marinum*, *M. avium* subsp. *Paratuberculosis*, *M. scrofulaceum*, *M. shimoidei*, *M. simiae*, *M. szulgai*, *M. ulcerans*, *M. xenopi*, *M. terrae*, *M. triviale*, *M. nonchromogenicum*, *M. abscessus*, *M. aurum*, *M. chelonae*, *M. chitae*, *M. fallax*, *M. flavescens*, *M. fortuitum* strain ATCC6841, *M. fortuitum* strain ATCC49403, *M. neoaurum*, *M.*  
 15 *peregrinum*, *M. phlei*, *M. senegalense*, *M. smegmatis*, *M. thermoresistibile*, and *M. vaccae*, in addition to the *Mycobacterium tuberculosis*.

The partial base sequences of the *rpoB* gene used to devise biotin-DGR8 and DGR9 primers are illustrated as the SEQ ID NOs 49-92 in Table 5.

[Table 5]

	Species	DGR9	DGR8
1	<i>M. tuberculosis</i>	tc gccgcgatca aggagt	gga ggtccgcgac gtgca
2	<i>M. africanum</i>	tc gccgcgatca aggagt	gga ggtccgcgac gtgca
3	<i>M. asiaticum</i>	tt gccgcgatca aggagt	gga agtgcgtgac gtgca
4	<i>Mycobacterium avium</i>	tg gcggcgatca aggagt	gga ggtccgcgac gtgca
5	<i>Mycobacterium bovis</i>	tc gccgcgatca aggagt	gga ggtccgcgac gtgca
6	<i>Mycobacterium bovis</i> BCG strain French 1173P2	tc gccgcgatca aggagt	gga ggtccgcgac gtgca
7	<i>Mycobacterium celatum</i> strain ATCC51131	tg gcggcgatca aggagt	cga ggtccgcgac gtgca
8	<i>Mycobacterium celatum</i> strain ATCC51130	tg gcggccatca aggagt	gga ggtccgcgac gtgca
9	<i>Mycobacterium gastri</i>	tc gccgccatta aggagt	gga ggtccgcgac gtgca
10	<i>Mycobacterium genavense</i>	tg gcggcgatca aggagt	cga ggtccgcgac gtgca
11	<i>Mycobacterium gordonae</i>	tc gccgcgatca aggagt	gga agtacgtgac gtgca
12	<i>Mycobacterium haemophilum</i>	tc gccgcgatca aggagt	gga agtacgtgac gtgca
13	<i>Mycobacterium interjectum</i>	tc gccgcgatca aggagt	cga ggtccgcgac gtgca
14	<i>Mycobacterium intermedium</i>	tc gccgcgatca aggagt	gga agtccgtgac gtgca

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15	<i>Mycobacterium intracellulare</i>	tc gccgcatca aggagt	gga ggtccgtgac gtcca
16	<i>Mycobacterium kansasii</i>	tc gccgcatca aggagt	gga ggtccgtgac gtcca
17	<i>Mycobacterium leprae</i>	tc gccgcatca aggagt	aga ggtccgtgac gtgca
18	<i>Mycobacterium malmoense</i>	tc gccgcatca aggagt	gga ggtccgtgac gtgca
19	<i>Mycobacterium marinum</i>	tt gccgcatca aggagt	gga agttcgtgac gtgca
20	<i>Mycobacterium avium</i> subsp. Paratuberculosis	tg gccgcatca aggagt	gga ggtccgtgac gtgca
21	<i>Mycobacterium scrofulaceum</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
22	<i>Mycobacterium shimoidei</i>	tt gccgcatca aggagt	gga agttcgtgac gtgca
23	<i>Mycobacterium simiae</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
24	<i>Mycobacterium szulgai</i>	tc gccgcatca aggagt	gga ggtccgtgac gtgca
25	<i>Mycobacterium ulcerans</i>	tt gccgcatca aggagt	gga agttcgtgac gtgca
26	<i>Mycobacterium xenopi</i>	tg gccgcatca aggagt	gga ggtccgtgac gtgca
27	<i>Mycobacterium terrae</i>	tc gccgcatca aggagt	tga ggtccgtgac gtgca
28	<i>Mycobacterium triviale</i>	tc gccgcatca aggagt	gga ggtccgtgac gtgca
29	<i>Mycobacterium nonchromogenicum</i>	tc gccgcatca aggagt	gga agttcgtgac gtgca
30	<i>Mycobacterium abscessus</i>	tg gccgcatca aggagt	cga ggtccgtgac gtgca
31	<i>Mycobacterium aurum</i>	tt gccgcatca aggagt	gga agttcgtgac gtgca
32	<i>Mycobacterium chelonae</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
33	<i>Mycobacterium chitae</i>	tg gccgcatca aggagt	cga ggtccgtgac gtgca
34	<i>Mycobacterium fallax</i>	tg gccgcatca aggagt	gga ggtccgtgac gtgca
35	<i>Mycobacterium flavescens</i>	tg gccgcatca aggagt	cga agttcgtgac gtgca
36	<i>Mycobacterium fortuitum</i> strain ATCC6841	tg gccgcatca aggagt	tga ggtccgtgac gtcca
37	<i>Mycobacterium fortuitum</i> strain ATCC49403	tg gccgcatca aggagt	tga ggtccgtgac gtcca
38	<i>Mycobacterium neoaurum</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
39	<i>Mycobacterium peregrinum</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
40	<i>Mycobacterium phlei</i>	tg gccgcatca aggagt	cga ggtccgtgac gtgca
41	<i>Mycobacterium senegalense</i>	tg gccgcatca aggagt	tga ggtccgtgac gtgca
42	<i>Mycobacterium smegmatis</i>	tg gccgcatca aggagt	cga ggtccgtgac gtgca
43	<i>Mycobacterium thermoresistibile</i>	tg gccgcatca aggagt	cga ggtccgtgac gtcca
44	<i>Mycobacterium vaccae</i>	tg gccgcatca aggaat	cga ggtccgtgac gtgca

In Table 6, the base sequences of the biotin-DGR8 and DGR9 fabricated as described above are illustrated and compared with the primer biotin-TR8 and TR9 of the prior application.

5

[Table 6]

PRIMER	BASE SEQUENCE
biotin -TR8	5' biotin t g c a c g t c g c g g a c c t c c 3'
TR9	5' t c g c c c g c g a t c a a g g a g t 3'
biotin-DGR8	5' biotin t g S a c g t c R c g N a c Y t c 3'
DGR9	5' t B g c S g c B a t Y a a g g a R t 3'

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Reference) B: c, t, g

N: c, t, g, a

R: g, a

S: c, g

5 Y: c, t

Embodiment 2-3: Polymerase chain reaction (PCR)

In order to find optimal conditions for PCR using the newly devised biotin-DGR8 and DGR9 primer set, reaction conditions were varied as follows:

A. Temperature program

10 a. at 94 °C for 5 minutes, at 94 °C for 30 seconds, at 47 °C for 30 seconds, and at 72 °C for 45 seconds

b. at 94 °C for 5 minutes, at 94 °C for 30 seconds, at 63 °C for 30 seconds, and at 72 °C for 45 seconds

15 c. at 94 °C for 5 minutes, at 94 °C for 30 seconds, at 64 °C for 30 seconds, and at 72 °C for 45 seconds

d. at 94 °C for 5 minutes, at 94 °C for 30 seconds, at 65 °C for 30 seconds, and at 72 °C for 45 seconds

20 The yield of PCR product using the biotin-DGR8 and biotin-DGR9 primers changed depending on the annealing temperature, as determined by a 2% agarose gel electrophoresis. At the annealing temperature of 64 or 65 °C, maximum yield was obtained with less amount of side products of different size that appeared at 47 or 63 °C.

B. Reaction cycle

25 Using the biotin-DGR8 and biotin-DGR9 primers, the amplified products of the polymerase chain reaction performed at 64 °C annealing temperature showed a single band with 35 cycles or 40 cycles as identified by a 2% agarose gel. The quantity of the amplified products was observed to be dependent on the reaction cycle, producing higher yield at 40 cycles. The most optimal PCR conditions were 64 °C and 40 cycles.

30 C. Primer concentration

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Using various concentrations of biotin-DGR8 and DGR9 primers, 50pmol, 100pmol, 500pmol, and 1000pmol, the polymerase chain reaction was performed at an annealing temperature of 64 °C for 40 cycles. It was observed that the yield increased within 50~100pmol concentration range but decreased  
5 with over 100 pmol. Therefore, biotin-DGR8 and DGR9 primer concentrations of 50-100pmol are considered appropriate.

Using the optimal conditions determined experimentally as described above, sixteen different Mycobacterial species were PCR amplified. FIG. 9A is a pictorial view showing a 2% agarose gel image of PCR amplified products  
10 under the reaction conditions disclosed in the prior application of the inventors, that is, at 63 °C and 35 cycles with biotin-TR8 and TR9 primers of 50pmol, and FIG. 9B is a pictorial view showing a 2% agarose gel image of PCR amplified products under the reaction conditions according to the present invention that is, at 64 °C and 40 cycles with biotin-DGR8 and biotin-DGR9 primers of 100pmol.

15 As illustrated in FIGs. 9A and 9B, the primer set of the present invention, biotin-DGR8 and DGR9, showed more efficient amplification of the rpoB 157bp region in all sixteen Mycobacterial species compared to biotin-TR8 and TR9 primers. The 157bp amplified products can be used as the target DNA for oligonucleotide chip analysis.

20 Embodiment 2-4: Drug-resistance diagnosis of sputum specimen

Eleven DNA samples directly extracted from sputum specimens of tuberculosis patients or suspected patients were obtained from the Department of Respiratory Diseases, Asan Medical Center (Seoul, Korea).

FIG. 10 is a pictorial view showing a 2% agarose gel image of PCR  
25 amplified products of sputum specimens under conditions as optimized in the embodiment 2-3 according to the present invention, i.e. 64 °C and 40 cycles with biotin-DGR8 and DGR9 primers of 100pmol. As shown in FIG. 10, Mycobacterium tuberculosis was not detected from #16 and #36 specimens. Nine specimens except for the above #16 and #36 specimens were determined as  
30 Mycobacterium tuberculosis positive. Thus, diagnosis by oligonucleotide chip



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to detect drug-resistance was performed using the oligonucleotide chip of FIG 7. As a result, it was determined that eight specimens including #6, #20, #26, #27, #29, #38, #41, #49 were wild-type, that is, rifampin sensitive strains, and #57 was a resistant strain having mutations on codons 509 and 513. FIGs. 11A through 11B show the images obtained from oligonucleotide chip experiments: #27 (wild-type) #57 (509, 513 mutant), respectively. DNA sequencing result of the eight wild-type strains agreed with the oligonucleotide chip result and #57 was determined to be a rifampin-resistance strain by drug-susceptibility test.

#### Embodiment 2-5: Random labeling incorporating Cyanine 5-dUTP

A new detection method has been devised to reduce assay time compared to the prior application method that uses streptavidin-R-phycoerythrin staining after PCR incorporating random biotin labeling. More specifically, PCR was performed with Cyanine 5-dUTP (NEN, U.S.A) instead of biotin-dUTP to incorporate Cyanine randomly to the amplified products, which does not require a separate staining procedure prior to scanning. Accordingly, an obvious visual result can be obtained speedily and simply. At the time of a polymerase chain reaction, a concentration of Cyanine 5-dUTP used equals to that of biotin-dUTP of the existing method, and it is preferable that DGR8 without biotin modification is used as a primer. The experimental conditions are the same as the existing method except that the staining process is omitted and that 633nm is used in fluorescence scanning

The parts which are not particularly described in the embodiments 2-1 through 2-5 are same as those of the embodiments 1-1 through 1-5, which will thus be omitted.

#### Industrial Applicability

Finally, as described above, the present invention can perform Mycobacterial species identification and drug-resistance detection with only a portion of Mycobacterial rpoB gene (157bp), simultaneously, speedily and accurately in large quantity, which can provide useful information in tuberculosis

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treatment so that an appropriate drug remedy can be given to the patients. Thus, at an early stage of the Mycobacterium infection, a proper administration of antibiotic agents and other necessary treatment can be provided to patients infected with mycobacteria.

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**Claims:**

1. A diagnosis kit for Mycobacterial species identification and drug-resistance detection comprising:

an oligonucleotide chip including a species identification probe  
5 comprised of species-specific DNA sequences of Mycobacterial rpoB gene (157bp), a Mycobacterial drug-resistance detection probe comprised of one or more modified codons of mycobacterial rpoB gene (157bp), and a contrast group probe comprised of wild-type sequences corresponding to each said drug-resistance detection probe; and

10 a marker for detecting a hybridization of said oligonucleotide chip and a specimen.

2. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1, wherein said species identification probe comprises SEQ ID NOs 5 through 12.

15 3. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1 or 2, wherein said Mycobacterium drug-resistance detection probe comprises one or more modified codons of 507-533 codons of rpoB gene.

4. The diagnosis kit for Mycobacterial species identification and drug-  
20 resistance detection of claim 3, wherein said drug-resistance detection probe is a rifampin-resistance detection probe.

5. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 4, wherein said rifampin-resistance detection probe comprises modified 511, 513, 516, 518, 522, 526, and 531 codons.

25 6. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 5, wherein said rifampin-resistance detection probe comprises probes of SEQ ID NOs 20 through 36.

7. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 5, wherein said rifampin-resistance probe further  
30 comprises modified 509, 533, and 524 codons.

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8. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 7, wherein said rifampin-resistance detection probe further comprises probes of SEQ ID NOs 41 through 46.

9. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 3 or 4, wherein said drug-resistance detection probe further comprises a rifabutin susceptibility detection probe.

10. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 9, wherein said rifabutin susceptibility detection probe comprises modified 516 and 526 codons.

11. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 10, wherein said rifabutin susceptibility detection probe comprises probes of SEQ ID NOs 22, 23, and 31 through 36.

12. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1 or 2, wherein said oligonucleotide chip is formed by a Schiff base reaction of each probe comprised of part of *rpoB* genes modified to contain an amine group and an aldehyde group induced on glass.

13. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1 or 2, further comprising a means for amplifying the DNAs of the specimen.

14. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 13, wherein said means comprises biotin-TR8 and TR9 primers of SEQ ID NOs 37 and 38 which amplify *rpoB* gene fragments (157bp) specifically.

15. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 13, wherein said means comprises biotin-DGR8 and DGR9 primers of SEQ ID NOs 47 and 48 which amplify *rpoB* gene fragments (157bp) specifically.

16. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 13, wherein said marker is a fluorescent material including said biotin-binding protein.

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17. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 16, wherein said fluorescent material is streptavidin-R-phycoerythrin.

18. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 13, wherein said marker is Cyinine 5-dUTP added in the polymerase chain reaction.

19. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1 or 2, wherein said each probe comprises  $T_{10}$  included at 5' as a spacer.

20. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 1 or 2, wherein said oligonucleotide chip further comprises a Mycobacterium complex probe which can detect whether a specimen is Mycobacterium.

21. The diagnosis kit for Mycobacterial species identification and drug-resistance detection of claim 20, wherein said tuberculosis bacteria group probe comprises probes of SEQ ID NOs 1 through 4.

22. A method of manufacturing a diagnosis kit for Mycobacterial species identification and drug-resistance detection comprising the steps of:

(a) modifying a species identification probe comprised of species-specific DNA sequences of Mycobacterial *rpoB* gene, a Mycobacterial drug-resistance detection probe comprised of one or more modified codons of Mycobacterial *rpoB* gene, and a contrast group probe comprised of wild-type sequences corresponding to each said drug-resistance detection probe to contain an amine group;

(b) inducing an aldehyde group on glass; and

(c) fabricating an oligonucleotide chip by affixing the modified probes on the glass with a Schiff base reaction, respectively.

23. The diagnosis kit manufacturing method of claim 22, further comprising the step of reducing a fixed imine bond formed in step (c) by  $\text{NaBH}_4$ .

24. A pair of primers comprising base sequences of SEQ ID NOs 47

- 30 -

and 48 which specifically amplifies rpoB gene fragments (157bp) of species belonging to Mycobacterium genus.

25. A method for Mycobacterial species identification and drug-resistance detection comprising the steps of:

- 5        amplifying rpoB gene fragments of specimen by a polymerase chain reaction (PCR) by using a pair of primers according to claim 24; and  
discriminating species by a fluorescent intensity corresponding to a particular species by using a diagnosis kit of claim 1.

26. The method for Mycobacterial species identification and drug-resistance detection of claim 25, wherein said PCR is performed at an annealing  
10        temperature of 64-65°C.

27. The method for Mycobacterial species identification and drug-resistance detection of claim 25 or 26, wherein said PCR is performed in a reaction period of 38-42.

15        28. The method for Mycobacterial species identification and drug-resistance detection of claim 25 or 26, wherein said PCR is performed in a primer concentration of 50-100pmol.

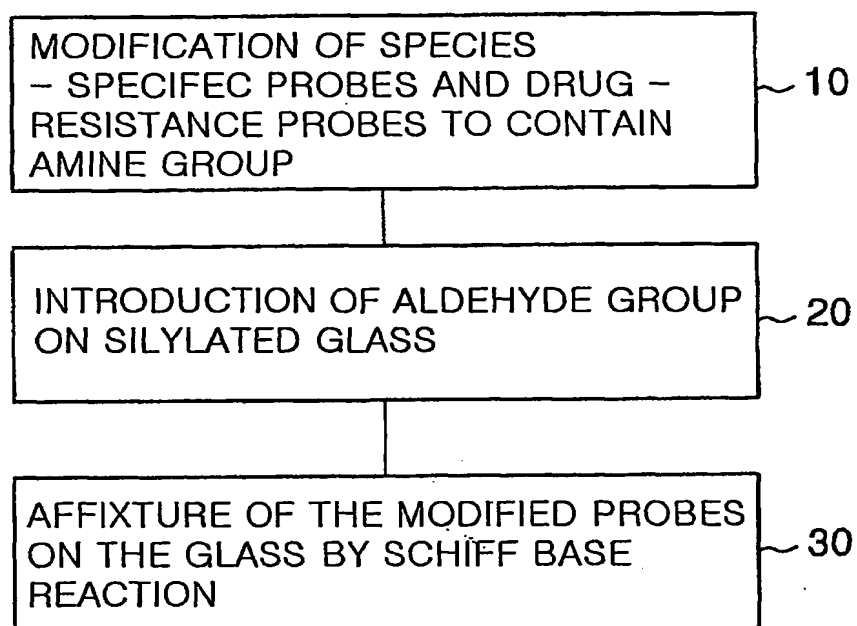
29. The method for Mycobacterial species identification and drug-resistance detection of claim 25, wherein said PCR further comprises a step of  
20        adding a Cynine 5-dUTP as a marker.

30. The method for Mycobacterial species identification and drug-resistance detection of claim 25, wherein said specimen is uncultured sputum, blood or cerebrospinal fluid of a patient.

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## DRAWING

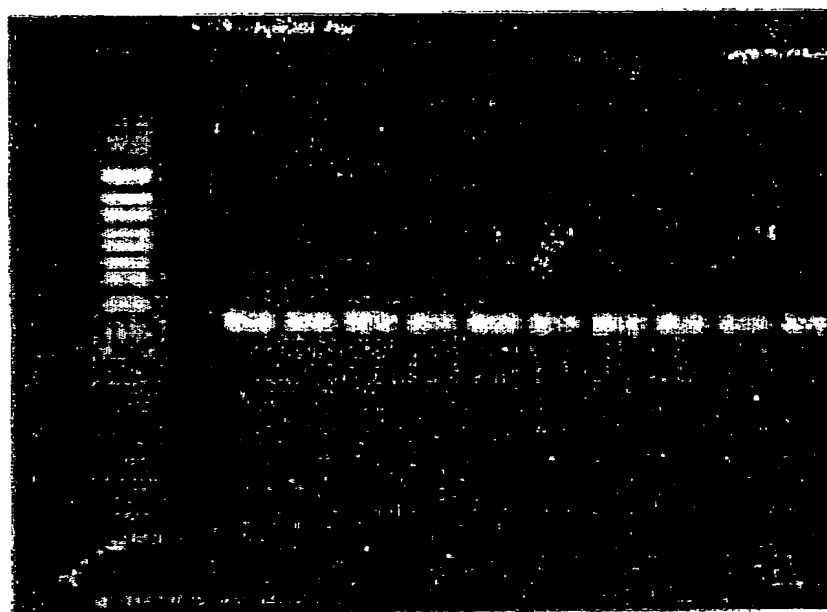
FIG. 1



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FIG. 2

M N 22 24 30 36 48 82 87 90 91 94

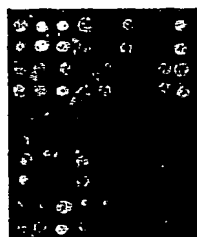


M:MAKER, N:NEGATIVE CONTROL  
NUMBER:CLINICAL ISOLATE NUMBER



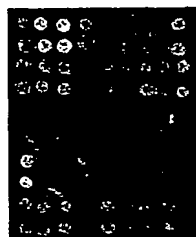
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FIG. 3a



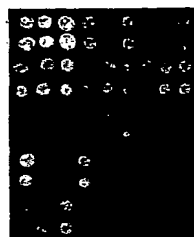
#22(mt516c)

FIG. 3b



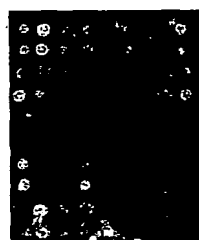
#24(mt526b/mt526e)

FIG. 3c



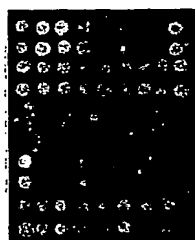
#30(mt531)

FIG. 3d



#36(mt516b)

FIG. 3e



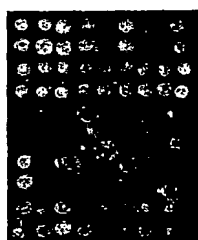
#48(mt526f)

FIG. 3f



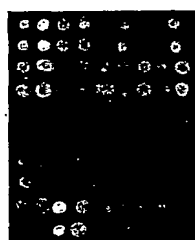
#82(mt531/mt516c)

FIG. 3g



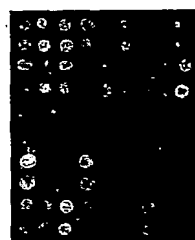
#87(mt531/mt516c)

FIG. 3h



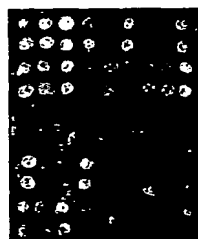
#90(mt516c/mt526d)

FIG. 3i



#91(mt531)

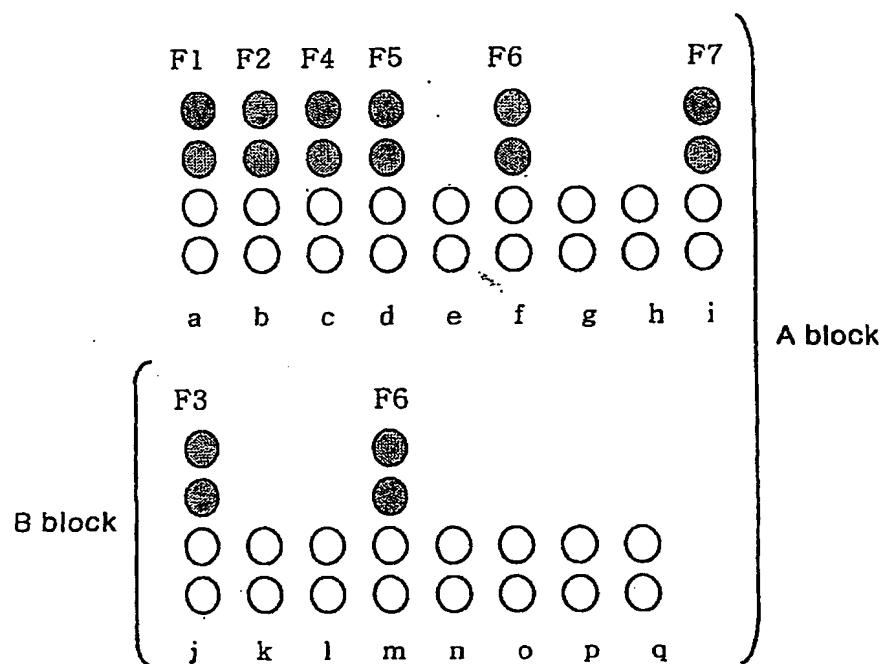
FIG. 3j



#94(mt511/mt531)

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FIG. 4

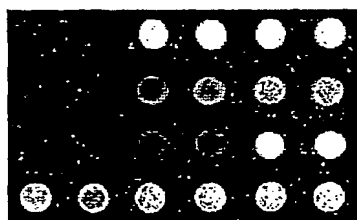


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FIG. 5

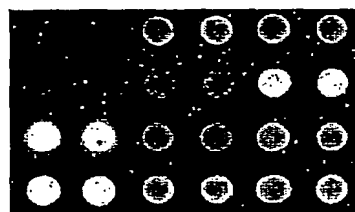
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		PROBE 6	PROBE 6	PROBE 2	PROBE 2
MEX	MEX	PROBE 7	PROBE 7	PROBE 3	PROBE 3
MC MIX	MC MIX	PROBE 8	PROBE 8	PROBE 4	PROBE 4

FIG. 6a



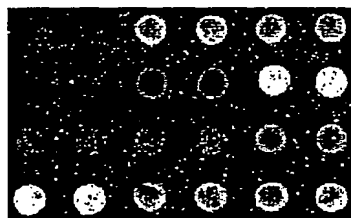
M.kansasii

FIG. 6b



M.fortuitum

FIG. 6c



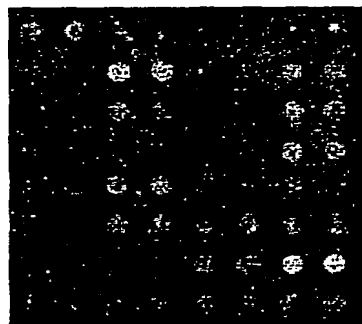
M.scrofalaceum

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FIG. 7

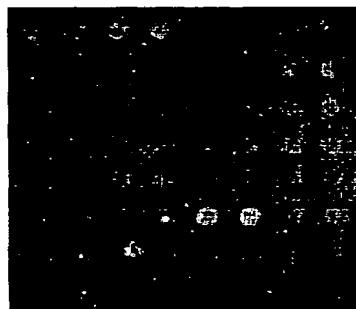
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Mex	Mex	Mt516a	Mt516a	Mt522	Mt522	Wt531	Wt531
Mt526h	Mt526h	Mt509	Mt509	Mt521	Mt521	Wt526	Wt526
Mt526g	Mt526g	Mt533	Mt533	Mt518	Mt518	Wt524	Wt524
Mt526f	Mt526f	Mt531	Mt531	Mt513	Mt513	Wt521	Wt521
Mt526e	Mt526e	Mt526c	Mt526c	Mt511	Mt511	Wt518	Wt518
Mt526d	Mt526d	Mt526b	Mt526b	Wt516	Wt516	Wt513	Wt513
Mt516c	Mt516c	Mt526a	Mt526a	Wt509	Wt509	Wt511	Wt511

FIG. 8a



mt516a

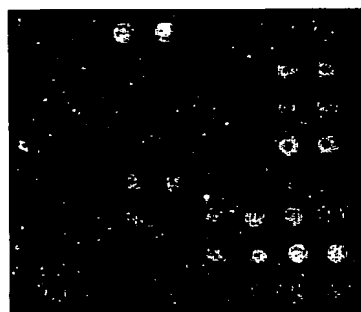
FIG. 8b



mt511

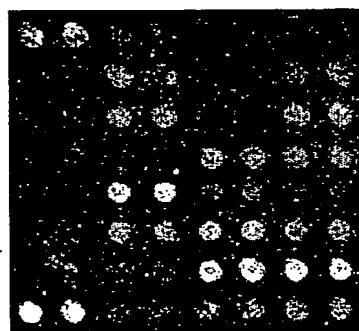
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FIG. 8c



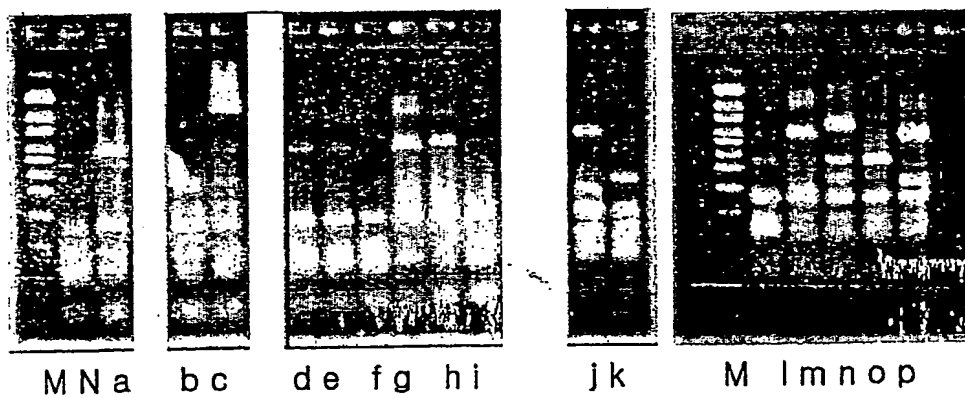
mt516b

FIG. 8d



mt531

FIG. 9a



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FIG. 9b

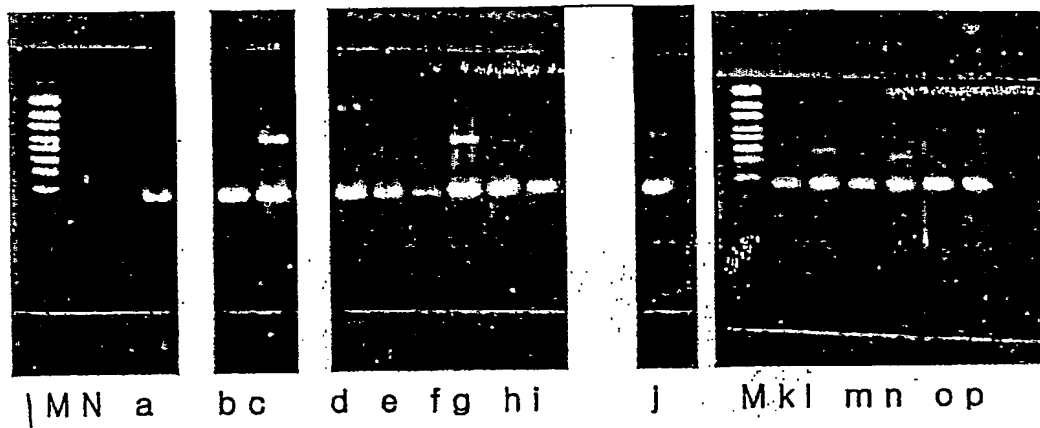
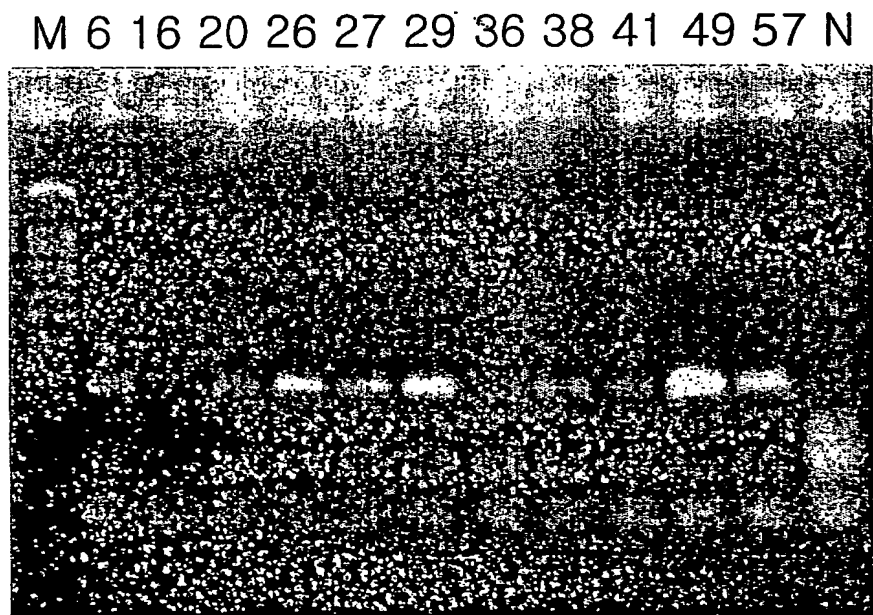


FIG. 10



M:MAKER, N:NEGATIVE CONTROL  
NUMBER:CLINICAL ISOLATE NUMBER

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FIG. 11a

#27(wt)

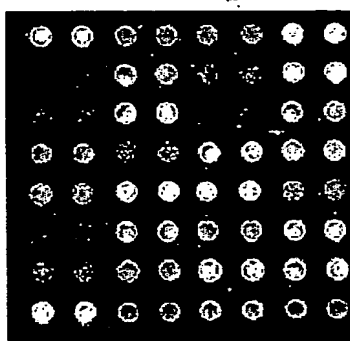
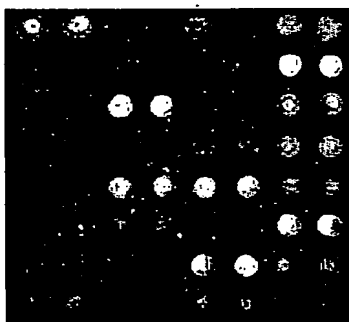


FIG. 11b

#57(mt509,513)



## Sequence Listing

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<120> diagnosis kit for Mycobacterium species identification and  
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<150> KR10-2000-0029369

<151> 2000-05-30

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<213> Mycobacterium tuberculosis

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<212> DNA

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## Sequence Listing

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<210> 8  
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## Sequence Listing

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<212> DNA

<213> Mycobacterium interjectum

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ggtctgtccc gcgagcgtg

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<210> 10

<211> 19

<212> DNA

<213> Mycobacterium xenopi

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<213> Mycobacterium chelonae, M. abscessus

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19

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19

## Sequence Listing

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## Sequence Listing

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## Sequence Listing

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<210> 24

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<212> DNA

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<210> 26

## Sequence Listing

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## Sequence Listing

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## Sequence Listing

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<400> 36  
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<210> 39  
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## Sequence Listing

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<213> Mycobacterium fortuitum, M.flavescens

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## Sequence Listing

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<212> DNA  
<213> Artificial Sequence

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belonged to Mycobacterium.

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## Sequence Listing

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 belonged to Mycobacterium.

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 <212> DNA  
 <213> Mycobacterium tuberculosis

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 aattcatgga ccagaacaac ccgctgtcgg ggttgaccca caagcgccga ctgtcggcgc 240  
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 cgcact 306

<210> 50  
 <211> 306  
 <212> DNA  
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## Sequence Listing

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 aattcatgga ccagaacaac ccgctgtcgg ggttgaccca caagcgccga ctgtcggcgc 240  
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 cgcaact 306

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 <212> DNA  
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## Sequence Listing

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agttcatgga ccagaacaac ccgtgtcggg ggctcaccca caagcgccgc ctgtcggcgc      240
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```

<210>      53
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<213>      Mycobacterium bovis

```

```

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tcaacatccg gccggtggtc gccgcgatca aggagttctt cggcaccagc cagctgagcc      180
aattcatgga ccagaacaac ccgtgtcggg ggttgacca caagcgccga ctgtcggcgc      240
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cgcaact                                           306

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<213>      Mycobacterium bovis BCG strain French 1173P2

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## Sequence Listing

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tggggcccg cggctctgtca cgtgagcgtg ccgggctgga ggtccgcgac gtgcaccgt      300
cgcaact                                          306

```

```

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<213>      Mycobacterium celatum strain ATCC51131

```

```

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agttcatgga ccagaacaac ccgctgtcgg ggttgacca caagcgccga ctgaacgcac      240
tgggcccggg tggctctgtc cgggagcggg cgggcctcga ggtgcgcgac gtgcaccga      300
gtcaact                                          306

```

```

<210>      56
<211>      306
<212>      DNA
<213>      Mycobacterium celatum strain ATCC51130

```

```

<400>      56
ttcgtaccgt cggtagagctg atccagaacc agatccgagt cggcatgtcc cgcattggagc      60
gggtggtccg cgagcggatg accactcagg acgtcgaggc gatcacgccg cagaccctga      120
tcaacatccg tcccgctgtg gcggcgatca aggagttctt cggcaccagc cagctctcgc      180

```

## Sequence Listing

```

agttcatgga ccagaacaac ccgctgtccg ggctgacca caagcgcgcg ctgaacgcac      240
tgggcccggg tggctgtgcc cgggagcggg cgggcctcga ggtgcgcgac gtgcacccga      300
gtcact                                                                    306

```

```

<210>      57
<211>      306
<212>      DNA
<213>      Mycobacterium gastri

```

```

<400>      57
tgcgcacggg gggcgagctg atccagaacc agatccgggt cggcatgtcc aggatggagc      60
gcgtcgtccg ggagcggatg accactcagg acgtcgaggc catcacgccg cagacgctga      120
tcaacattcg cccggtggtc gccgccatta aggagtctt cggcaccagc cagctgtcgc      180
agttcatgga ccagaacaac ccgctgtcgg gctgacca caagcgccgg ctttcggcgc      240
tgggccccgg cggctgttca cgtgagcgtg cgggctgga ggtccgcgac gtgcacccgt      300
cgcaact                                                                    306

```

```

<210>      58
<211>      306
<212>      DNA
<213>      Mycobacterium genavense

```

```

<400>      58
tgcgcacggg gggcgatctg atccagaacc agatccgggt cggcatgtcg cggatggagc      60
gggtgggtccg tgagcggatg accactcagg acgtcgaggc catcacgccg cagaccctga      120
tcaacatccg tccggttgtg gcggcgatca aggagtctt cggcaccagc cagctctcgc      180
agttcatgga ccagaacaac ccgctgtcag gtctcaccca caagcgccgg ttgtcggcgc      240

```

## Sequence Listing

tggggcccggg cggctctgtcc cgtgagcggg cgggcctcga ggtccgcgac gtgcacccgt 300

cgcaact 306

<210> 59

<211> 306

<212> DNA

<213> *Mycobacterium gordonae*

<400> 59

tgcgcacccgt gggcgagctg atccagaacc agatccgggt cggcatgtcc cggatggagc 60

gcgtcgtgcg cgagcggatg accactcagg acgtcgaggc catcacgccg cagaccctga 120

tcaacatccg gccggctcgtc gccgcgatca aggagtctt cggcaccagc cagctctcgc 180

agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240

tggggcccggg tggctctgtcc cgtgagcgtg cgggtctgga agtacgtgac gtgcacccgt 300

cgcaact 306

<210> 60

<211> 306

<212> DNA

<213> *Mycobacterium haemophilum*

<400> 60

tgcgcacccgt gggcgagctg atccagaacc agatccgggt cggcatgtcc cggatggagc 60

gcgtcgtgcg cgagcggatg accactcagg acgtcgaggc catcacgccg cagaccctga 120

tcaacatccg gccggctcgtc gccgcgatca aggagtctt cggcaccagc cagctctcgc 180

agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240

tggggcccggg tggctctgtcc cgtgagcgtg cgggtctgga agtacgtgac gtgcacccgt 300



## Sequence Listing

---

cgcaact 306

<210> 61  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium interjectum

<400> 61  
 tgcgcaccgt cggtagctg atccagaacc agatccgggt cggcatgtcc aggatggagc 60  
 gcgtcgtccg ggagcggatg accaccacagg acgtcgaggc gatcacgccg cagacgctga 120  
 tcaacatccg gccggtcgtc gccgcgatca aggagttctt cggcaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg gcctcaccca caagcgccgc ctgtcggcgc 240  
 tgggcccggg cggctgtgtcc cgcgagcggg ccggcctoga ggtccgcgac gtgcacccga 300  
 accact 306

<210> 62  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium intermedium

<400> 62  
 tgcgtaccgt cggcgagctg atccagaacc agatccgggt cggcatgtcc cgcattggagc 60  
 gcgttgtccg cgagcggatg accactcagg acgtcgaggc catcacgccg cagaccttga 120  
 tcaacatccg gccggtggtc gccgcgatca aggagttctt cggcaccagc cagctctcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgt 240  
 tgggcccggg tggctgtgtc cgtgagcgtg ccgggctgga agtccgtgac gtgcacccgt 300  
 cgcaact 306

## Sequence Listing

<210> 63  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium intracellulare

<400> 63  
 tgcgcaccgt gggtagctg atccagaacc agatccgggt cggcatgtcg cggatggagc 60  
 gcgtcgtccg cgagcggatg accacgcagg acgtcgaggc catcacgccg cagaccctga 120  
 tcaacatccg gccggtcgtc gccgcgatca aggagttctt cggcaccagc cagctgagcc 180  
 agttcatgga ccagaacaac ccgctgtccg gtctgaccca caagcgcgcg ctctcggcgc 240  
 tgggccccgg cggctctgtcc cgtgagcgcg ccggcctgga ggtccgtgac gtccaccct 300  
 cgcaact 306

<210> 64  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium kansasii

<400> 64  
 tgcgcaccgt gggtagctg atccagaacc agatccgggt cggcatgtcg cggatggagc 60  
 gcgtcgtccg cgagcggatg accacgcagg acgtcgaggc catcacgccg cagaccctga 120  
 tcaacatccg gccggtcgtc gccgcgatca aggagttctt cggcaccagc cagctgagcc 180  
 agttcatgga ccagaacaac ccgctgtccg gtctgaccca caagcgcgcg ctctcggcgc 240  
 tgggccccgg cggctctgtcc cgtgagcgcg ccggcctgga ggtccgtgac gtccaccct 300  
 cgcaact 306

## Sequence Listing

<210> 65  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium leprae*

<400> 65  
 tgcgcacggt cggcgaattg atccagaacc agatccgggt cggatatgtcg cggatggagc 60  
 ggggtggtccg ggagcggatg accaccagg acgtcgaggc gatcacgccg cagacgctga 120  
 tcaatatccg tccggtggtc gccgctatca aggaattctt cggcaccagc cagctgtcgc 180  
 agttcatgga tcagaacaac cctctgtcgg gcttgaccca caagcgccgg ctgtcggcgc 240  
 tgggcccggg tggtttgtcg cgtgagcgtg ccgggctaga ggtccgtgac gtgcaccctt 300  
 cgcaact 306

<210> 66  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium malmoeense*

<400> 66  
 tgcgcacggt cggggagctg atccagaacc agatccgcgt cggcatgtcg cggatggagc 60  
 gcgtcgtccg ggagcggatg accaccagg acgtcgaggc gatcacgccg cagacgctga 120  
 tcaacatccg gccggtggtc gccgcgatca aggagttctt cggcaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg ggctgaccca caagcgccgg ctgtcggcgc 240  
 tgggcccggg tggctctgtcg cgtgagcgtg ccggcttgga ggtccgtgac gtgcaccgt 300  
 cgcaact 306

<210> 67  
 <211> 306

## Sequence Listing

<212> DNA

<213> *Mycobacterium marinum*

<400> 67

tgcgcacggt gggtagactg atccagaacc agatccgggt cggcatgtcg cggatggagc	60
gggtgggtccg ggagcggatg accacccagg acgtcgaggc gatcacgccg cagacgctga	120
tcaacatccg tccggtcgtt gcggcgatca aggagttctt cggaaccagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctctccg gtctcaccca caagcgccgc ctctcggcgc	240
tggggccggg cggctctgtc cgtgagcgcg ccggtctgga agttcgtgac gtgcacccgt	300
cgcaact	306

<210> 68

<211> 306

<212> DNA

<213> *Mycobacterium avium* subsp. *paratuberculosis*

<400> 68

tgcgcacggt cggtagactg atccagaacc agatccgggt cggcatgtcc cggatggagc	60
gcgtcgtccg cgagcggatg accacccagg acgtcgaggc catcacgccg cagaccctga	120
tcaacatccg tccggtcgtg gcggcgatca aggagttctt cggcaccagc cagttgtccc	180
agttcatgga ccagaacaac ccgctgtcgg ggctcaccca caagcgccgc ctgtcggcgc	240
tggggccggg tggctctgtc cgggagcggt ccgggctgga ggtccgcgac gtgcacccgt	300
cccact	306

<210> 69

<211> 306

<212> DNA

<213> *Mycobacterium scrofulaceum*

## Sequence Listing

<400> 69  
 tgcgcaccgt cggtagctg atccagaacc agatccgggt cggcctgtcg cgtatggagc 60  
 gtgtcgtcgc tgagcgcgtg accacccagg acgtcgaggc gatcaccccg cagaccctga 120  
 tcaacatccg ccccgctcgtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240  
 tgggccccgg cggctctgtcc cgtgagcgcg ccggccttga ggtccgcgac gtgcactcca 300  
 gccact 306

<210> 70  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium shimoidei*

<400> 70  
 tgcgcacggt gggtgagctg atccagaacc agatccgggt cggcatgtcg cggatggagc 60  
 gggtggtccg ggagcggatg accacccagg acgtcgaggc gatcacgccg cagacgctga 120  
 tcaacatccg tccggtcgtt gccgcgatca aggagttctt cggaaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtccg gtctcaccca caagcgccgc ctctcggcgc 240  
 tggggccggg cggctctgtcc cgtgagcggt ccgggctgga agttcgtgac gtgcacccgt 300  
 cgact 306

<210> 71  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium simiae*

<400> 71

## Sequence Listing

---

tgcgacccgt gggtagctg atccagaacc agatccgggt cggcctgtcc cgcattggagc	60
gcgtcgtgcg tgagcggatg accacccagg acgtcgaggc gatcacgccg cagaccctga	120
tcaacatccg tcccgctgtg gcggcgatca aggagttctt cggtagcagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctttcgg gtctgaccca caagcgtcgc ctgtcggcgc	240
tgggccccgg cggctgtgtcc cgtgagcgtg ccggccttga ggtccgcgac gtgcacgcca	300
gccact	306

<210> 72  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium szulgai

tgcgacccgt gggcgagttg atccagaacc agatccgggt cggcatgtcc cggatggagc	60
gcgtcgtgcg cgagcggatg accacccagg acgtcgaggc gatcacgccg cagaccctga	120
tcaacatccg gcccgctgtc gccgcgatca aggagttctt cggcaccagc cagctctcgc	180
agttcatgga ccagaacaac ccgctctcgg gtctgaccca caagcggcgt ctgtccgctc	240
tggggccggg cggctgtgtcc cgtgagcggg ccgggcttga ggtccgtgac gtgcacccgt	300
cgact	306

<210> 73  
 <211> 306  
 <212> DNA  
 <213> Mycobacterium ulcerans

tgcgacccgt gggtagctg atccagaacc agatccgggt cggcatgtcg cggatggagc	60
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## Sequence Listing

---

gggtgggtccg ggagcggatg accacccagg atgtcgaggc gatcacgccg cagacgctga	120
tcaacatccg tccggtcgtt gccgcgatca aggagttctt cggaaccagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctctccg gtctcaccca caagcgccgc ctctcggcgc	240
tggggccggg cggctctgtc cgtgagcgcg ccggtctgga agttcgtgac gtgcaccgt	300
cgcact	306

<210> 74  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium xenopi*

<400> 74 tgcgcacggt cggcgagctg atccaaaacc agatccgggt cggcatgtcg aggatggagc	60
gggtgggtccg cgagcggatg accactcagg acgtcgaggc gatcaccccg cagacctga	120
tcaacatccg cccgtgggtg gccgcgatca aggagttctt cggcaccagc cagctctcgc	180
agttcatgga tcagaacaac ccgctgtcgg ggctcaccca caagcgccgc ctctcggcgc	240
ttggtccggg cggctctgtc cgcgagcggg ccgggctgga ggtccgtgac gtgcactcga	300
gccact	306

<210> 75  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium terrae*

<400> 75 tgcgcacggt gggtagctg atccagaacc agatccgggt cgggttgtcc cggatggagc	60
gtgtgggtccg cgagcggatg accacccagg acgtcgaggc catcacgccg cagaccctga	120

## Sequence Listing

---

tcaacatccg cccggtggtc gccgcgatca aggagtcttt cggcaccagc cagctctcgc	180
agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgccgg ctgtcggcgc	240
tgggccccgg tggctgttcc cgtgagcgtg ccgggcttga ggtccgtgac gtgcaccgt	300
cccact	306

<210> 76  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium triviale*

<400> 76 tgcgcaccgt cggggagttg atccagaacc agatccgggt cgggctgtcc cggatggagc	60
gggtggtgcg cgagcggatg accacccagg atgtcgaggc gatcacgccg cagaccctga	120
tcaacatccg cccggtggtc gccgcgatca aggagtcttt cggcaccagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctgtcgg ggctgaccca caagcgccgg ctgtcggcgc	240
tggggccccg cgggctctcc cgggagcggg ccgggcttga ggtccgcgac gtgcaccca	300
gccact	306

<210> 77  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium nonchromogenicum*

<400> 77 tgcgcaccgt ggtgagctg atccagaacc agatccgggt cgggctgtcc cggatggagc	60
gcgtggtccg cgagcggatg accacccagg acgtcgaggc catcacgccg cagaccctga	120
tcaacatccg cccggtggtc gccgccatca aggaattctt cggcaccagc cagctgtcgc	180



## Sequence Listing

```

agttcatgga ccagaacaac ccgctgtcag gtctgaccca caagcggcgt ctgtcggcgc      240
tgggccccgg tggctctgctg cgtgagcgcg ccggcctgga agttcgtgac gtgcaccctg      300
cccaact                                     306

```

```

<210>      78
<211>      306
<212>      DNA
<213>      Mycobacterium abscessus

```

```

<400>      78
tgcgtaccgt cggcgagctg attcagaacc agatccgggt cggcctgtcc cgtatggagc      60
gcgtcgtgctg tgagcgcatg accacgcagg acgtcgaggc gatcaccccg cagaccctga      120
tcaacatccg tcccgtcgtg gccgcgatca aggagttctt cggaaccagc cagctgtcgc      180
agttcatgga ccagaacaac ccgctgtcgg gcctgaccca caagcgtcgt ctgtcggcgc      240
tgggccccgg tggctctgacc cgtgaccgcg ccggcctcga ggtccgcgac gtgcaccctt      300
cgcaact                                     306

```

```

<210>      79
<211>      306
<212>      DNA
<213>      Mycobacterium aurum

```

```

<400>      79
tgcgcaccgt gggcgagttg atccagaacc agatccgggt cggcatgtcc cggatggagc      60
gcgtcgtccg cgagcggatg accactcagg acgtcgaggc gatcacgccg cagaccctga      120
tcaacatccg gccggtcgtt gccgcgatca aggagttctt cggcaccagc cagctctcgc      180
agttcatgga ccagaacaac ccgctttcgg gtctgaccca caagcggcgc ctgtcggcgc      240

```

## Sequence Listing

---

tgggccccgg cggctctgtcc cgtgagcgtg ccggcctgga agtgcgtagac gtgcaccct 300

cgcaact 306

<210> 80

<211> 306

<212> DNA

<213> *Mycobacterium chelonae*

<400> 80

tgcgtaccgt cggcgagctg atccagaacc agatccgggt cggcctgtcg cgtatggagc 60

gcgtcgtgcy tgagcgcagc accactcagg acgtcgaggc gatcaccccg cagaccctga 120

tcaacatccg tcccgtcgtg gcggcgatca aggagttctt cggaaccagc cagctgtcgc 180

agttcatgga ccagaacaac ccgctttcgg gtctgaccca caagcgtcgt ctgtcggctc 240

tgggccccgg tggctctgacc cgtgaccgcy ctggccttga ggtccgcgac gtgcaccct 300

cgcaact 306

<210> 81

<211> 306

<212> DNA

<213> *Mycobacterium chitae*

<400> 81

tgcgcaccgt gggtagctg atccagaacc agatccgggt cggcctgtcc cgcattggagc 60

gcgtcgtgcy cgagcggatg accaccagg acgtcgaggc catcacgccg cagaccctga 120

tcaacatccg tcccgtcgtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc 180

agttcatgga ccagaacaac ccgctgtccg ggctgaccca caagcgtcgt ctctcggcgc 240

tcgggccccg cggctctgtcc cgtgagcgcy ccggctctga ggctcgtgac gtgcaccct 300

## Sequence Listing

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	cgcaact	306
<p>&lt;210&gt; 82</p> <p>&lt;211&gt; 306</p> <p>&lt;212&gt; DNA</p> <p>&lt;213&gt; Mycobacterium fallax</p>		
<p>&lt;400&gt; 82</p>	<p>tgcgcaccgt gggcgagctg atccagaacc agatccgggt cggcctgtcc cggatggagc</p> <p>gcgtcgtccg cgagcggatg accaccagg acgtcgaggc gatcaccccg cagaccctga</p> <p>tcaacatccg tcccgtggtg gcggcgatca aggagtctt cgggaccagc cagctgtcgc</p> <p>agttcatgga ccagaacaac ccgtgtcgg gcctgaccca caagcgccg ctgtccgcgc</p> <p>ttggccccgg cggctctgtcc cgtgagcgcg ccggcctgga ggtccgcgac gtgcacgcca</p> <p>gccact</p>	<p>60</p> <p>120</p> <p>180</p> <p>240</p> <p>300</p> <p>306</p>
<p>&lt;210&gt; 83</p> <p>&lt;211&gt; 306</p> <p>&lt;212&gt; DNA</p> <p>&lt;213&gt; Mycobacterium flavescens</p>		
<p>&lt;400&gt; 83</p>	<p>tgcgcaccgt cggcgagctg atccagaacc agatccgggt cggcctgtcg cggatggagc</p> <p>gcgtcgtccg tgagcggatg accaccagg acgtcgaggc gatcacgccg cagaccctga</p> <p>tcaacatccg tcccgtcgtg gcggcgatca aggagtctt cggtagctcg cagctgtcgc</p> <p>agttcatgga ccagaacaac ccgtgtcgg gtctgaccca caagcgccg ctgtccgcgc</p> <p>tgggccccgg tggctctgtcc cgtgagcgcg ccggcctcga agtccgtgac gtgcaccgt</p> <p>cgcaact</p>	<p>60</p> <p>120</p> <p>180</p> <p>240</p> <p>300</p> <p>306</p>

## Sequence Listing

---

<210> 84  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium fortuitum* strain ATCC6841

<400> 84  
 tgcgcaccgt gggcgagctg atccagaacc agatccgctg cggcctgtcc cgcattggagc 60  
 gcgtcgtgcg tgagcgcatt accacccagg acgtcgaggc gatcaccccg cagaccctga 120  
 tcaacatccg tcccgctctg gcggcgatca aggagttctt cggaacgtcg cagctgtcgc 180  
 agttcatgga tcagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240  
 tgggcccccg cggctctgtcc cgtgagcgcg ccggccttga ggtccgcgac gtccactcgt 300  
 cgcact 306

<210> 85  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium fortuitum* strain ATCC49403

<400> 85  
 tgcgcaccgt gggcgagctg atccagaacc agatccgggt cggcctgtcc cgcattggagc 60  
 gcgtcgtgcg tgagcgcatt accacccagg acgtcgaggc gatcaccccg cagaccctga 120  
 tcaacatccg tcccgctctg gcggcgatca aggagttctt cggaacgtcg cagctgtcgc 180  
 agttcatgga tcagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240  
 tgggcccccg cggctctgtcc cgtgagcgcg ccggccttga ggtccgcgac gtccactcgt 300  
 cgcact 306

<210> 86

# Sequence Listing

---

<211> 306  
 <212> DNA  
 <213> *Mycobacterium neoaurum*

<400> 86  
 tgcgcaccgt gggtagctg atccagaatc agatccgggt cggcctgtcg cgcattggagc 60  
 gggtagctg cgcgcctatg accacccagg acgtcgaggc gatcaccgag cagaccctga 120  
 tcaacatccg tcccgtagtg gcggcgatca aggagttctt cgggaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240  
 tgggccccgg tggtagtcc cgtgagcgtg ccggacttga ggtccgcgac gtgcactcca 300  
 gccact 306

<210> 87  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium peregrinum*

<400> 87  
 tgcgcaccgt cggtagctg atccagaacc agatccgggt cggcctgtcg cgtatggagc 60  
 gtgtcgtgag tgagcgcctg accacccagg acgtcgaggc gatcaccgag cagaccctga 120  
 tcaacatccg ccccgtagtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc 180  
 agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgtcgt ctgtcggcgc 240  
 tgggccccgg cggtagtcc cgtgagcgtg ccggacttga ggtccgcgac gtgcactcca 300  
 gccact 306

<210> 88  
 <211> 306  
 <212> DNA

## Sequence Listing

<213>     *Mycobacterium phlei*

<400>     88

tgcgacccgt cggcgagctg atccagaacc agatccgggt cggcctgtcg cgtatggagc	60
gcgtcgtgcg cgagcgcatg accacccagg acgtcgaggc gatcacgccg cagaccctga	120
tcaacatccg tcccgtcgtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctgtcgg gtctgaccca caagcgccgc ctgtcggcgc	240
tgggccccgg cggctctgtcc cgtgagcgcg ccggccttga ggtccgcgac gtgcaccaca	300
gccact	306

<210>     89

<211>     306

<212>     DNA

<213>     *Mycobacterium senegalense*

<400>     89

tgcgacccgt gggtagagctg atccagaacc agatccgggt cggcctgtcc cgcattggagc	60
gcgtcgtgcg tgagcggatg accacccagg acgtcgaggc gatcacgccg cagaccctga	120
tcaacatccg tcccgtcgtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc	180
agttcatgga ccagaacaac ccgctttcgg gtctgaccca caagcgtcgc ctgtcggcgc	240
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gccact	306

<210>     90

<211>     306

<212>     DNA

<213>     *Mycobacterium smegmatis*

## Sequence Listing

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 tcaacatccg tcccgctcgtg gcggcgatca aggagttctt cggcaccagc cagctgtcgc 180  
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 gccact 306

<210> 91  
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 <213> *Mycobacterium thermoresistibile*

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 tcaacatccg ccccgctcgtg gcggcgatca aggagttctt cggcaccagc cagctgagcc 180  
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 cgact 306

<210> 92  
 <211> 306  
 <212> DNA  
 <213> *Mycobacterium vaccae*

<400> 92  
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## Sequence Listing

gtgtcgtccg cgagcggatg accacccagg acgtcgaggc gatcactccg cagacctga	120
tcaacatccg tcccgtcgtg gcggcgatca aggaattctt cggcaccagc cagctgtcgc	180
agtcatgga ccagaacaac ccgtgtcgg gtctgacctt caagcgtcgc ctgtcggcgc	240
tgggccccgg cggctctgtc cgtgagcgcg ccggcctcga ggtccgcgac gtgcactcca	300
gccact	306



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR01/00904

**A. CLASSIFICATION OF SUBJECT MATTER****IPC7 C12Q 1/68**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patent and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Registry file, CAPLUS, WPI, Medline, BIOSIS in STN

"mycobacteri? and rpoB and drug and resistance and probe and hybridiz?"

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Thomas R. Gingeras et al. 'Simultaneous Genotyping and Species Identification Using Hybridization Pattern Recognition Analysis of Generic Mycobacterium DNA Arrays' In: Genome Research, 1998, Vol. 8, Issue 5, pp. 435-448	1,3-5, 13, 16-18, 20, 22
X	Troesch A. et al. 'Mycobacterium species identification and rifampin resistance testing with high-density DNA probe array' In: Journal of Clinical Microbiology, 1999, Vol. 37, No.1, pp. 49-55	1,3-5, 13, 16-18, 20, 22
P, X	EP 1076099 A2 (NISSHINBO INDUSTRIES, INC) 14 FEBRUARY 2001	1-30
A	Victor T. C. et al. 'Detection of Mutation in Drug Resistance Genes of Mycobacterium Tuberculosis by a Dot-blot Hybridization Strategy' In: Tubercle and Lung Disease, 1999, Vol. 79, No. 6, pp. 343-348	1-30
A	Rossau, R. et al. 'Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of Mycobacterium tuberculosis complex and its resistance to rifampin', 1997, Vol. 41, No. 10, pp. 2093-2098	1-30

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

28 SEPTEMBER 2001 (28.09.2001)

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Name and mailing address of the ISA/KR

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